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Intragranular Colocalization of Arginine Vasopressin- and Angiotensin II-Like Immunoreactivity in the Hypothalamo-Neurohypophysial System of the Goldfish, *Carassius auratus*

Chifumi Yamada[1], Shinobu Noji[2,3], Seiji Shioda[4], Yasumitsu Nakai[4] and Hideshi Kobayashi

Research Laboratory, Zenyaku Kogyo Co., Ltd., Tokyo 178, [2]Department of Biology, Faculty of Science, Toho University, Chiba 274, [4]Department of Anatomy, Showa University School of Medicine, Tokyo 142, Japan

ABSTRACT—In the preoptic nucleus (PON) of the goldfish, *Carassius auratus*, four types of cells were observed under a light microscope: cells showing colocalization of immunoreactive angiotensin II (ANG II) and immunoreactive agrinine vasopressin (AVP), cells with only ANG II-like immunoreactivity, cells with only AVP-like immunoreactivity and cells with neither immunoreactivity. Under an electron microscope, only two types of nerve terminals were found in the neurohypophysis: those showing immunoreactivity of both antisera and those with neither immunoreactivity. No terminals showing alternative immunoreactivity could be found. The discrepancy in these findings obtained by light and electron microscopes is discussed. In nerve terminals reactive to both antisera, an immunogold technique indicated the presence of neurosecretory granules with colocalization of immunoreactive ANG II and AVP, granules with only ANG II-like immunoreactivity and granules showing only AVP-like immunoreactivity. The AVP-like immunoreactivity observed in the PON and the neurohypophysis is considered due to arginine vasotocin.

INTRODUCTION

A renin-angiotensin system in the brain has been demonstrated biochemically and pharmacologically in mammals [1]. Immunohistochemically, angiotensin II (ANG II) and arginine vasopressin (AVP) have been shown to be present in the same neurons of the supraoptic, paraventicular and suprachiasmatic nuclei of the rat [2–4]. However, intragranular colocalization of these peptides in these neurons has not been studied.

In the present study, the colocalization of immunoreactive ANG II and immunoreactive AVP was examined in neurosecretory cells of the preoptic nucleus (PON) of the goldfish, *Carassius anratus*. Further, intragranular colocalization of these substances was electron microscopically examined in axon terminals in the neurohypophysis.

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[1] Present address: Department of Physiology, National Defense Medical College, Tokorozawa 359, Japan

[3] Present address: Department of Clinical Laboratories, Keio University Hospital, Tokyo 160, Japan

MATERIALS AND METHODS

Antisera

The following antisera were used for light microscopy. Antiserum to ANG II was raised in rabbit against synthetic Asp[1]-Ileu[5]-ANG II (Protein Research Foundation, Osaka) by Yamaguchi [5]. The complete cross-reactivities of this antiserum with Asp[1]-Val[5]-ANG II [5] and Asn[1]-Val[5]-ANG II (unpublished data) were demonstrated by radioimmunoassay. Antiserum to arginine AVP was raised in rabbit against synthetic AVP (Protein Research Foundation, Osaka) and cross-reacted with arginine vasotocin (AVT) at 42% and with

oxytocin (OXT) at only 3.5% [6].

For electron microscopy, the ANG II antiserum was the same as that used for the light microscopic experiment. AVP antiserum raised against synthetic AVP in rabbit (UCB Bioproducts, Belgium) and having complete cross-reactivity with AVT and less than 0.003% cross reactivity with oxytocin or mesotocin was used.

Light microscopy

Twenty five goldfish, *Carassius auratus* (about 10 cm in total length) were obtained commercially. After decapitation, brains with the pituitary or brains alone were quickly removed and fixed in Bouin's solution overnight. Tissue was dehydrated through a series of ethanol, cleared in xylol and embedded in paraffin. Four μm thick sagittal sections were made and mounted on slides. To examine the colocalization of immunoreactive ANG II and AVP, two consecutive sections were immunostained, one with ANG II and the other with AVP antiserum.

Deparaffinized preparations were immunostained by the peroxidase-anti-peroxidase (PAP) method of Sternberger *et al.* [7]. Incubation was performed as follows: (1) in 0.3% H_2O_2 for 30 min at room temperature (RT), (2) in ANG II antiserum (1 : 1000) or AVP antiserum (1 : 2000) overnight at 4°C, (3) in goat anti-rabbit IgG (GAR; Polysciences Inc., Warrington, Pennsylvania; 1 : 200) for 90 min at RT, (4) in peroxidase-anti-peroxidase (PAP; Dako Corp., Copenhagen or Cappel Laboratories, West Chester, Pennsylvania; 1 : 200) for 90 min at RT, and (5) in 0.02% 3,3′-diaminobenzidine in 0.05 M Tris buffer (pH 7.6) containing 0.006% H_2O_2 for 10–15 min at RT. To rinse the preparations and dilute the antisera, 0.1 M phosphate buffer saline (pH 7.2) containing 0.3% Triton X-100 was used.

For the control, immunostaining was conducted using the following sera instead of the primary antisera: normal rabbit serum (NRS; Polysciences Inc., Warrington, Pennsylvania; 1 : 1600), ANG II antiserum preabsorbed with Asn^1-Val^5-ANG II (Hypertensin, Ciba; 20, 100 μg/ml diluted antiserum), ANG II antiserum preabsorbed with AVT (Protein Research Foundation, Osaka; 20 μg/ml diluted antiserum), AVP antiserum preabsorbed with Asn^1-Val^5-ANG II (20 μg/ml diluted antiserum), the primary antisera preincubated with 1% bovine serum albumin(BSA) and AVP antiserum preabsorbed with AVT (20, 100 μg/ml diluted antiserum). AVP antiserum was preabsorbed with AVT but not AVP, since, as is well known, neurosecretory cells produce AVT but not AVP in teleosts.

Electron microscopy

Ten goldfish (each about 8 cm in total length) were obtained from a commercial source. They were anesthetized with 0.01% ethyl m-aminobenzoate methanesulfonate (MS222) and perfused with a mixture of paraformaldehyde (4%) and glutaraldehyde (0.4%) in 0.05 M phosphate buffer (PB; pH 7.2). The pituitary of each specimen was removed and fixed in the same fixative for 2–3 hr at 4°C. This was followed by rinsing in 0.1 M Millonig PB and postfixation in 2% OsO_4 in 0.1 M Millonig PB for 1.5 hr at 4°C. All tissue was subsequently dehydrated through a series of ethanol, transferred to propylene oxide and embedded in an Epon-Araldite mixture. Ultrathin sections were cut and mounted on 200-mesh nickel grids.

Ultrathin sections were stained by a double immunogold technique. First, one face of a section was incubated in (1) saturated sodium metaperiodate for 30 min at RT, (2) 1% egg albumin in 0.01 M PBS (pH 7.2) for 10 min at RT, (3) AVP antiserum (1 : 16000) overnight at 4°C, and (4) colloidal gold labeled GAR (1 : 20; gold particles of about 5 nm in diameter) for 90 min at RT. Next, another face was incubated in the same way, but immunostained with ANG II antiserum (1 : 1000) overnight at 4°C, and colloidal gold labeled GAR (1 : 10; gold particles of about 15 nm in diameter) for 90 min at RT. Immunostained sections were stained further with both uranyl acetate and lead citrate, and examined with Hitachi HS-9 and HU-12A electron microscopes.

For the control, ANG II antiserum preabsorbed with either Asn^1-Val^5-ANG II or AVT (each 10 μg/diluted antiserum), and AVP antiserum preabsorbed with AVT, ANG II or isotocin (Protein Research Foundation, Osaka) (each 10 μg/diluted antiserum) were used as primary antisera.

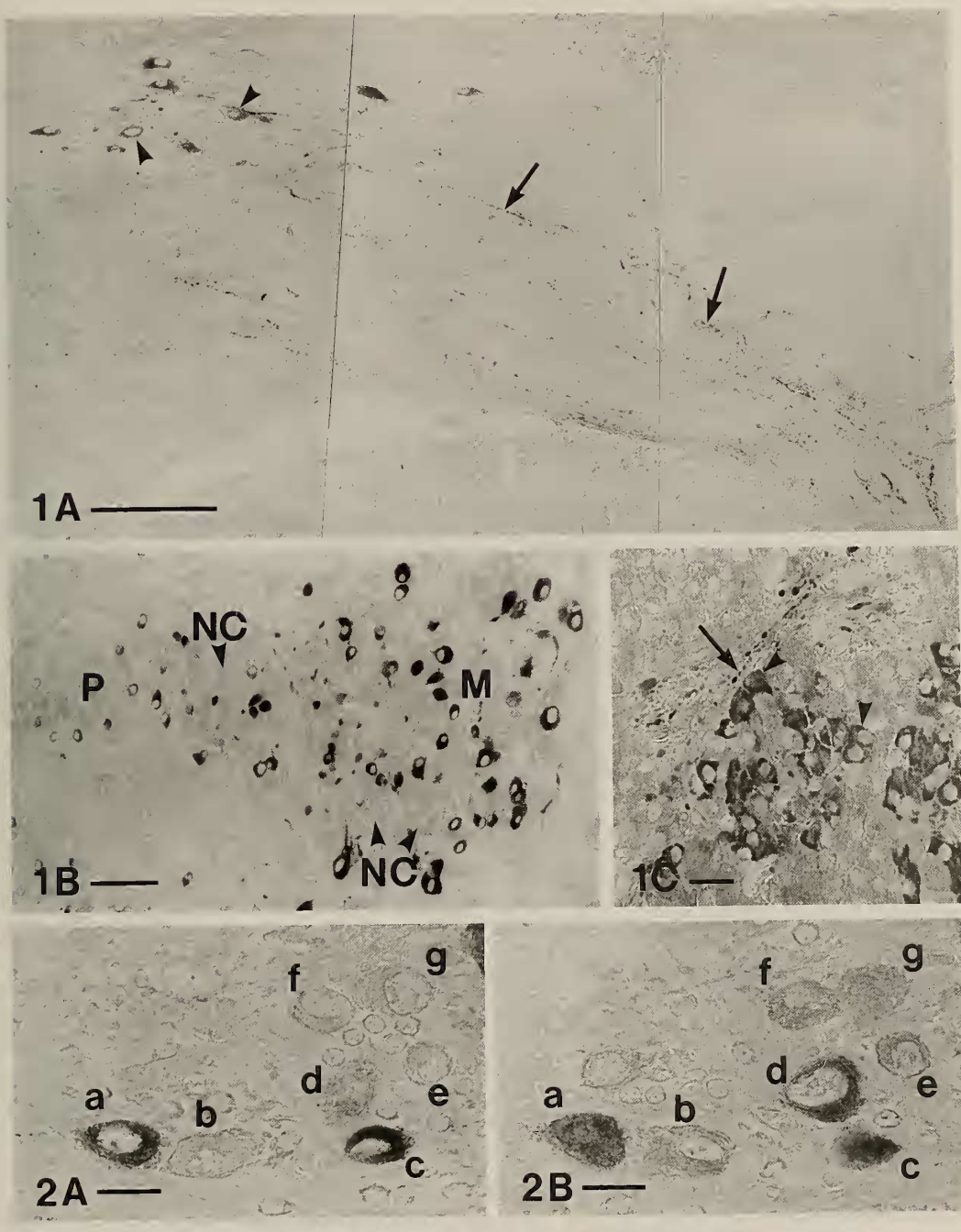

FIG. 1. A: ANG II-like immunoreactive cells in the PON (arrow-heads) and their fibers (arrows) extending to the neurohypophysis in the hypothalamus of the goldfish, *Carassius auratus*. Bar=100 μm. B: ANG II-like immunoreactive cells in magnocellular (M) and parvocellular groups (P) of the PON. In both groups, many non-immunoreactive cells (NC) were found. Bar=50 μm. C: ANG II-like immunoreactive cells (arrowheads) in the pars distalis and immunoreactive fibers (arrow) extending to the pars distalis from the PON. Reaction of fibers to ANG II antiserum was abolished by preabsorption of the serum with ANG II, but not that of the cells. Bar=20 μm.

FIG. 2. Two consecutive sections of a PON region of the goldfish. Immunostaining with ANG II (A) and AVP (B) antisera. Cells a, c and d were reactive to both antisera; cells b, e, f and g were reactive only to AVP antiserum. Bar=20 μm.

RESULTS

Light microscopy

ANG II and AVP antisera, preabsorbed with synthetic ANG II and AVT, respectively, showed no indication of immunoreaction. However, ANG II immunoreaction in the cells of the pars distalis was not abolished by ANG II antiserum preabsorbed with ANG II. Immunoreaction of these cells is considered nonspecific. Immunostaining by NRS also failed to indicate immunoreaction. Other control sera used did not abolish immunoreactions. Immunoreaction to ANG II antiserum in the brain may thus be considered specific to ANG II and the immunoreaction to AVP antiserum observed in the brain is considered due not to AVP but to AVT. That teleostean neurosecretory neurons produce AVT but not AVP supports these considerations.

Immunoreactivity to ANG II antiserum was observed in the cells of the magnocellular and parvocellular groups of the preoptic nucleus (PON) (Figs. 1A, B, 2A) as well as to AVP antiserum (Fig. 2B). The colocalization of ANG II- and AVP-like immunoreactivity was evident in many neurons (Fig. 2). Certain neurons possessed only AVP-like immunoreactivity (Fig. 2), while others, only ANG II-like immunoreactivity; the number of the latter was very small. Neurons

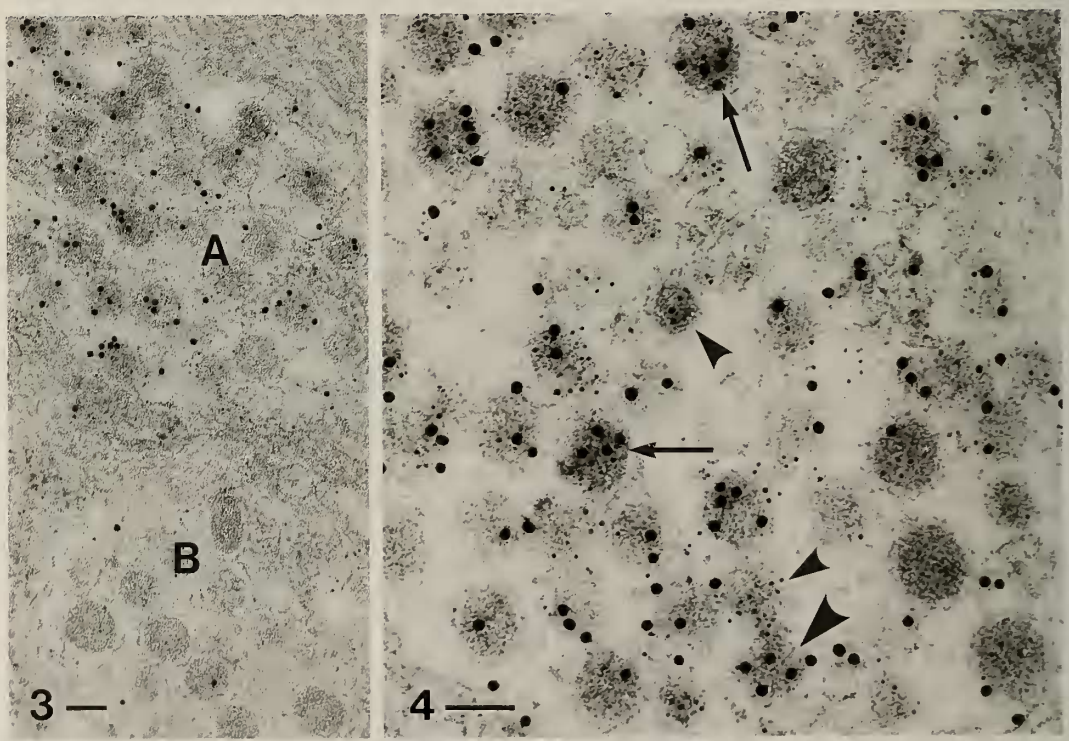

FIG. 3. Ultrastructural localization of ANG II- and AVP-like immunoreactivity in the neurohypophysis of a goldfish. Large colloidal gold particles (diameter, about 15 nm) and small colloidal gold particles (diameter, about 5 nm) demonstrate ANG II- and AVP-like immunoreactivity, respectively. A nerve terminal (A) contained both immunoreactivities while another, (B), neither. Bar = 100 nm.

FIG. 4. Intragranular localization of ANG II- and AVP-like immunoreactivity in the same nerve terminal of the neurohypophysis of a goldfish. Large colloidal gold particles (diameter, about 15 nm) and small colloidal gold particles (diameter, about 5 nm) demonstrate ANG II- and AVP-like immunoreactivity, respectively. In some neurosecretory granules, both immunoreactivities could be detected (arrows). Some granules show only ANG II-like immunoreactivity (large arrowheads) while others, only AVP-like immunoreactivity (small arrowheads). Bar = 100 nm.

showing no immunoreaction to either antiserum were also present. The fibers with either ANG II- or AVP-like immunoreactivity extended as far as to the neurohypophysis (Fig. 1A) and pars distalis (Fig. 1C). In the pars distalis, immunoreaction to ANG II antiserum was observed in the cells and fibers (Fig. 1C). The reaction of the fibers in the pars distalis was abolished by ANG II antiserum preabsorbed with ANG II, but that of the cells was not abolished because of its nonspecificity. The cells of the pars intermedia were not stained by either antiserum.

Electron microscopy

In control experiments, immunoreaction was abolished by preabsorption of ANG II and AVP antisera with Asn^1-Val^5-ANG II and AVT, respectively. Immunoreaction to ANG II antiserum was not abolished by preabsorption of ANG II antiserum with AVT, nor was that to AVP antiserum preabsorbed with ANG II or isotocin.

Electron microscopy indicated a number of granules and synaptic vesicle-like structures to be present in nerve terminals in the neurohypophysis. Two types of axon terminals were observed, those with both ANG II- and AVP-like immunoreactivity and those with neither immunoreactivity (Fig. 3). In the former terminals, the three following kinds of granules (about 80 nm in diameter) were detected: 1) granules showing both ANG II- and AVP-like immunoreactivity, indicated by large and small gold particles, respectively, 2) granules showing only ANG II-like immunoreactivity and 3) granules showing only AVP-like immunoreactivity (Fig. 4). The ratio of these kinds of granules differed for each terminal. Some granules in the terminals with both immunoreactivities showed greater ANG II-like immunoreactivity than AVP-like immunoreactivity or visa versa.

DISCUSSION

In the present study, immunoreaction to AVP antiserum was frequently observed in the cells of the magnocellular and parvocellular groups of the PON and in the neurohypophysis. This reaction may possibly be due to AVT, since the AVP antiserum used in the present experiment was demonstrated to cross-react with AVT, and also, it is known that one of the neurohypophysial hormones in teleosts is AVT but not AVP. Thus, in the following description, AVT was used instead of AVP.

Most fibers of ANG II-like immunoreactive neurons in the magnocellular and parvocellular groups extended as far as to the neurohypophysis, as in the case of the rat [8, 9]. Some invaded the pars distalis of the adenohypophysis, as well as AVT fibers. It would thus seem that the hypothalamo-hypophysial nervous system of ANG II is present in the goldfish, although its function has yet to be clarified.

The colocalization of ANG II- and AVT-like immunoreactivity was observed in the perikarya of many neurosecretory neurons of the PON. It has also been demonstrated that immunoreactive ANG II and AVP colocalized in the neurons of the paraventricular, supraoptic and suprachiasmatic nuclei in the rat [2, 4]. Further, the intragranular colocalization of immunoreactive ANG II and immunoreactive AVT in some axon terminals of the neurohypophysis was found in the present study. It would appear that both peptides are simultaneously released from these terminals into the capillaries. In the rat, ANG II has been shown to stimulate ACTH release from the adenohypophysis as well as AVP [10] and ANG II and AVP to potentiate the ACTH-releasing activity of corticotropin-releasing factor (CRF) [11 for ANG II, 12 for AVP]. In the goldfish, ANG II and AVT stimulate the release of ACTH [13]. It should thus be reasonable to conclude that ANG II and AVT, following their simultaneous release from the same terminal, may potentiate the ACTH-releasing activity of CRF in this fish.

The colocalization of AVP and CRF in the neurons of the paraventricular nucleus and in the fibers in the median eminence has been reported in mammals [14–17]. In teleosts, this has also been shown in some neurons of the PON [13, 18, 19]. The present authors noted ANG II-, AVT- and CRF-like immunoreactivity in the same neurons in the PON of the goldfish (unpublished data). Thus, ANG II, AVT and CRF may be released simultaneously from the same neurons. These peptides possibly exert a synergistical effect on the release

of ACTH from the adenohypophysis or ANG II and AVT may modulate the release of CRF.

By light microscope, four types of the nerve cells were observed in the PON: 1) cells with both immunoreactive ANG II and AVT, 2) cells with only immunoreactive AVT, 3) cells with only immunoreactive ANG II, and 4) cells without any immunoreaction. By electron microscope, however, only two types of nerve terminals were found in the neurohypophysis: 1) terminals showing immunoreactive ANG II and AVT, and 2) terminals showing no immunoreactivity. The discrepancy with respect to cell type number as determined using these different microscopes may be due to variation in the amount of storage of these peptides in the cell bodies and in the terminals. Immunoreactivity in cells containing the peptides in very small amounts would not be detected by light microscopy, leading to the erroneous conclusion that there are four cell types. However, both light and electron microscopy also indicate the presence of cells and nerve terminals containing neither ANG II- nor AVT-like immunoreactivity. These neurons may contain neuropeptides other than ANG II or AVT.

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